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Philipp Gerhardt

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Microbiology & Public Health
Michigan State University
East Lansing, MI 48824-1101

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13. ABSTRACT *(Maximum 200 words)* Bacterial spores possess extraordinary resistance against destruction by heat and other agents, so extreme safeguards must be taken to prevent spore-caused infections and food spoilage. A determination of the physicochemical bases and physiological mechanisms accounting for sporal heat resistance is the goal of this project. The project involves collaboration by teams of investigators headed by Philipp Gerhardt at Michigan State University and by Robert E. Marquis at the University of Rochester. The titles of six published research reports convey their content: heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation; heat shock affects permeability and resistance of Bacillus stearothermophilus spores; low heat resistance of B. sphaericus spores correlated with high protoplast water content; spectrophotometric determination of refractive index increment for bacterial cells; heat resistance correlated with DNA content in B. megaterium spores; compact structure of cortical peptidoglycans from bacterial spores. The titles of four published review essays convey their content: the refractory homeostasis of bacterial spores; turgor pressure, sporulation, and the physical properties of cell walls; minerals and bacterial spores; spore thermoresistance mechanisms.

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MECHANISMS OF RESISTANCE IN MICROBIAL SPORES

PHILIPP GERHARDT

Department of Microbiology and Public Health
Michigan State University
East Lansing, MI 48824-1101

20 December 1990

STATEMENT OF THE PROBLEM STUDIED

Bacterial spores possess extraordinary resistance against destruction by heat and other deleterious agents, so extreme safeguards must be taken in medicine and industry to cope with this property. For example, wound dressings and instruments must be sterilized by autoclaving to prevent the introduction of tetanus or gangrene spores, and foods must be pressure-heat canned to prevent clostridial spoilage and the survival of toxigenic botulism spores. Such preliminary measures could be greatly lessened if spore resistance could be circumvented.

Bacterial spores furthermore exemplify the general biological phenomenon of dormancy or cryptobiosis, the deathlike state of suspended activity first demonstrated in 1702 by Leeuwenhoek with rotifers and nematodes. Cryptobiosis occurs also in the primitive tardigrade animals, the cysts and larvae of certain crustaceans and insects, the seeds of plants, the spores of fungi, and certain viruses. But in bacteria the phenomenon occurs in extreme degree: for example, spores of thermoactinomycetes have been revived after survival in archaeological deposits for almost 2,000 years. Bacterial spores thus provide an excellent unicellular, procaryotic model for studying the extended maintenance of the organized cellular and molecular structure that characterizes living organisms.

Despite these practical and theoretical implications, and a century of research, how spores achieve resistance remains undefined. In seeking to explain the resistance of spores, it is necessary to discern both the physicochemical bases for stabilization of essential structures and macromolecules and the physiological mechanisms for attainment of the stabilization. For example, a low water content in the sporal protoplast might account in part for the stabilization, and an inward pressure exerted by the cortex expressing water from the protoplast might account for the attainment of this state. Furthermore, multiple bases and mechanisms probably prevail. To attain thermostability a labile molecule or structure must be immobilized in some manner, such as by removal of the surrounding water, by restriction of motion in molecular structure imposed by mechanisms such as contraction and crossbonding, or by intercalation of a cation such as calcium and stabilization by a chelating molecule such as dipicolinate. It may be necessary to account not only for the resistance of the vital structures and macromolecules within the protoplast but also that of the enzymes outside the protoplast which are required for initiation of the germination process. Moreover, probable differences between the nature of resistance to one type of agent (e.g., moist heat) and another (e.g., dry heat or radiation) must be considered.

A key to solution of the problem appears to lie with unique in situ properties which are lost when the spore is analyzed by the usual extraction and fractionation techniques. The crucial properties appear essentially biophysical in character. Consequently, it appears necessary to examine these properties by use of probes that do not destroy the structural and molecular configurations conferring resistance. We have developed methods for nondestructively probing the spore interior, particularly by use of dielectric, light refractometric and molecular permeation techniques. We have

also explored the use of other biophysical techniques, some quite fruitful such as electron probe x-ray microanalysis and some less so such as ultrasonic analysis.

This biophysical approach has been combined in the project with the use of genetic, biochemical and microbiological techniques. The isolation of mutants and chemical divestment has provided spores that are defective or denuded of the coat-outer membrane complex (as evidenced by lysozyme susceptibility) so that only the inner membrane remains functional. This technique has enabled quantification of protoplast water content within fully hydrated dormant spores, first by differential permeability measurements and then by buoyant density determinations that need only small amounts of impure spores. Another breakthrough in methodology has been provided by the development of techniques to completely demineralize dormant spores, and then to remineralize them with specific cations.

These complementary approaches are brought together by long-standing collaboration between the teams of investigators in laboratories at Michigan State University and the University of Rochester. Each laboratory shares its special skills and facilities with the other, the same spore models and preparations are used as much as possible to enable correlations, and ideas are exchanged regularly. The project thus essentially embodies two USARO contracts rather than one.

Furthermore, this collaborative project is part of an international inter-disciplinary program for research on spore resistance. This international program has included exchange of individual scientists for short-term and long-term working visits, and sharing of spore preparations. The program has also included seminar-workshop meetings in 1977, 1980, 1984 and 1985 followed by publications of progress reports in special issues of Spore Newsletter. The Co-Principal Investigators of this project have served as co-convenors and co-editors for these initiatives. For example, the Co-Principal Investigators organized a seminar-workshop on spore resistance mechanisms held in conjunction with the International Congress of Microbiology at Manchester, U.K. in September of 1986.

The present project was a renewal of a prior USARO contract, supplemented by a major equipment award. Substantial progress has been accomplished toward accomplishment of the general goal, which is to explain how bacterial spores are so highly resistant to heat.

PARTICIPATING SCIENTIFIC PERSONNEL

At Michigan State University: Philipp Gerhardt, Teofila C. Beaman, H. Stuart Pankratz, Brian H. Belliveau, Thomas R. Corner, J. T. Greenamyre.

At University of Rochester: Robert E. Marquis, Gary R. Bender, Edwin L. Carstensen.

SUMMARY OF MOST IMPORTANT EXPERIMENTAL RESULTS

Bacterial spores possess extraordinary resistance against destruction by heat and other deleterious agents, so extreme safeguards must be taken to prevent spore-caused infections, food spoilage and food poisoning. A determination of the physicochemical bases and physiological mechanisms accounting for sporal heat resistance (SHR) is the goal of this project. The rationale of approach is to employ biophysical probes that are invasive but not destructive to the cellular and molecular configurations conferring resistance in intact spores. Genetic, biochemical and microbiological techniques are also employed. The project involves collaboration by teams of investigators at two universities. Substantial progress has been accomplished during the contract period, including publication of six original research reports and four review articles.

By use of our previously described method to measure protoplast water content (PWC) by means of buoyant density sedimentation in a permeating medium, the PWC was determined with 29 types among 8 Bacillus species spanning a 3,000-fold range in SHR, which was altered by acid demineralization and specific remineralization and also by thermal adaptation. These factors caused changes in PWC and thereby changes in SHR between limits of 57 and 28% in PWC. Outside these limits, however, these factors correlated independently with SHR. Thus, protoplast dehydration, mineralization and thermal adaptation all contribute to SHR in a complex relationship. However, dehydration of the protoplast is the only determinant necessary and sufficient in itself for the elevated heat resistance characteristic of bacterial spores.

In a further study, an increased DNA content of model digenomic spores was shown to confer greater SHR, but only in the usually small digenomic fraction of a population. Thus, DNA content is apparently not a general and usual determinant of SHR.

The amount of cortical peptidoglycan (CPG), however, has long been known to affect SHR, and is relatable to the extent of protoplast dehydration. The chemical structure of this crucial spore component has long been believed to be loosely cross-linked with high net electrical charge, and to function as a contractile or expansive structure expressing water from the protoplast during spore formation. Contrary to this prevalent belief, CPG isolated by chemical extraction of decoated spores was found to have few free amino groups, indicative of a high level of peptide cross-linking; low mineral contents, indicative of low levels of net negative charge; and low dextran-impermeable volumes, indicative of compact cortical structures. Moreover, CPG had little capacity to swell or shrink in response to changes in environmental pH value. Overall, the data indicated that the CPG sacculus is designed to serve primarily as a restraining structure rather than as a contractile or expansive element.

Although found inapplicable to spores, a method was developed to use an ordinary spectrophotometer for obtaining the refractive index increment of bacterial cells, enabling determination of their solids (and water) content by immersion refractometry.

Heat-activated spores of Bacillus stearothermophilus were found to be separable into two distinct populations by use of buoyant density

centrifugation with a selected gradient medium. The fully activated spores in the one fraction became permeabilized at the outer membrane (and, thus, lysozyme susceptible), and were slightly less heat resistant than the original dormant spores. The partly activated spores in the other fraction remained lysozyme resistant but became substantially more heat resistant than the original dormant spores. This phenomenon of super-resistance may involve either in situ induction or selection of a preexisting subpopulation.

LIST AND ABSTRACTS OF PUBLICATIONS

Beaman, T.C., and P. Gerhardt. 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl. Environ. Microbiol. 52:1242-1246.

Twenty-eight types of lysozyme-sensitive spores among seven *Bacillus* species representative of thermophiles, mesophiles, and psychrophiles were obtained spanning a 3,000-fold range in moist-heat resistance. The resistance within species was altered by demineralization of the native spores to protonated spores and remineralization of the protonated spores to calcified spores and by thermal adaptation at maximum, optimum, and minimum sporulation temperatures. Protoplast wet densities, and thereby protoplast water contents, were obtained by buoyant density sedimentation in Nycodenz gradients (Nyegaard and Co., Oslo, Norway). Increases in mineralization and thermal adaptation caused reductions in protoplast water content between limits of ca. 57 and 28% (wet weight basis), and thereby correlated with increases in sporal heat resistance. Above and below these limits, however, increases in mineralization and thermal adaptation correlated with increases in sporal resistance independently of unchanged protoplast water contents. All three factors evidently contributed to and were necessary for heat resistance of the spores, but dehydration predominated.

Gerhardt, P. 1988. The refractory homeostasis of bacterial spores, p. 41-49. In R.G. Board et al. (ed.), Homeostatic mechanisms in microorganisms. FEMS Symposium No. 4, Bath University Press, UK. (*No abstract in the article*).

Beaman, T.C., H.S. Pankratz, and P. Gerhardt. 1988. Heat shock affects permeability and resistance of Bacillus stearothermophilus spores. Appl. Environ. Microbiol. 54:2515-2520.

Heat shock of dormant spores of *Bacillus stearothermophilus* ATCC 7953 at 100 or 80°C for short times, the so-called activation or breaking of dormancy, was investigated by separating the resulting spores by buoyant density centrifugation into a band at 1.240 g/ml that was distinct from another band at 1.340 g/ml, the same density as the original spores. The proportion of spores at 1.240 g/ml became larger when the original dormant spores were heated for a longer period of time, but integument-stripped dormant spores were quickly and completely converted to spores with a band at 1.240 g/ml. The spores with bands at both 1.240 and 1.340 g/ml were germinable faster than the original dormant spores and thus were considered to be activated. The spores with a band at 1.240 g/ml, which were considered to be fully activated, were apparently permeabilized, with a resulting complete depletion of dipicolinic acid, partial depletion of minerals, susceptibility to lysozyme action, permeation of the gradient medium, changed structural appearance in electron micrographs of thin-sectioned spores, and partly decreased heat resistance (D_{100} = 453 min) compared with the original dormant spores (D_{100} = 760 min). However, the fully activated spores with a band at 1.240 g/ml, although devoid of dipicolinic acid, still were much more resistant than germinated spores or vegetative cells (D_{100} = 0.1 min). The spores with a band at 1.340 g/ml, which were considered to be partly activated, showed no evidence of permeabilization and were much more heat resistant (D_{100} = 1,960 min) than the original dormant spores. This phenomenon of super-resistance may involve either in situ induction or selection of a preexisting subpopulation.

Marquis, R.E. 1988. Turgor pressure, sporulation, and the physical properties of cell walls, p. 21-32. In P. Actor, L. Daneo-Moore, M.L. Higgins, M.R.J. Salton and G.D. Shockman (ed.). Antibiotic inhibition of bacterial surface assembly and function. Amer. Soc. Microbiol., Washington, DC. (*No abstract in the article*).

Marquis, R.E. 1989. Minerals and bacterial spores, p. 247-274.. In T.J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley and Sons, New York. (*No abstract in the article*).

Beaman, T.C., H.S. Pankratz, and P. Gerhardt. 1989. Low heat resistance of Bacillus sphaericus spores correlated with high protoplast water content. FEMS Microbiol. Lett. 58:1-4.

The low heat resistance ($D_{100} = 0.554$ min, $z = 13.4$ °C) of dormant lysozyme-sensitized spores of *Bacillus sphaericus* 9602 was correlated with a low protoplast wet density (1.305 g/ml) equivalent to a high protoplast water content (61.0%, wet weight basis). These values for these unusual spores were consistent with those correlated previously in 28 spore types of seven other species.

Gerhardt, P., and R.E. Marquis. 1989. Spore thermoresistance mechanisms, pp. 43-63. In I. Smith, R.A. Slepecky and P. Setlow (ed.). Regulation of prokaryotic development. Amer. Soc. Microbiol., Washington, DC.

Experimental evidence enables us now to define quantitatively the main physicochemical determinants of heat resistance and to envisage an increasingly clear view of the physiological processes by which thermoresistance is attained during sporogenesis and maintained during dormancy of bacterial spores. Spores are resistant by about 40°C more than their corresponding vegetative cells. Dehydration of the protoplast is the only determinant necessary and sufficient in itself for the elevated level of heat resistance characteristic of spores. The protoplast water content of fully hydrated spores varies between limits of about 57 to 28% on a wet-weight basis in lysozyme-sensitive spores and presumably also in lysozyme-resistant spores. The processes for attaining protoplast dehydration are not fully understood, but we propose a novel concept of potassium-coupled osmotic egress of water at an early stage of sporogenesis. The maintenance of protoplast dehydration requires an intact cortex, but not coat nor exosporium. The extent of protoplast dehydration depends on the relative amount of cortical peptidoglycan, which is now shown to be tightly cross-linked.

A second determinant of spore heat resistance is protoplast mineralization, mostly by calcium. Mineralization affects heat resistance independently at the upper and lower limits of protoplast dehydration, but between them is reflected in changed water content. Mineralization is apparently attained by active transport through the sporangial membrane and passive flow into the developing spore. Although often associated with minerals, DPA is apparently not necessary for attaining heat resistance, though may function in retaining it.

A third determinant of spore heat resistance is thermal adaptation, which mostly is genetically inherent. However, increasing the temperature of sporulation can impose additional heat resistance, with or without affecting protoplast dehydration. Heat resistance is increased temporarily in spores partly activated by sublethal heat shock, but is decreased in fully activated spores.

At the molecular level, as evidenced especially by dielectric measurements, the spore protoplast contains complexes of small molecules, macromolecules, and supramolecular structures so compacted, bonded, and dehydrated that they become immobilized and thus thermostabilized.

Corner, T.R., T. C. Beaman, J.T. Greenamyre, and P. Gerhardt. 1990. Spectrophotometric determination of refractive index increment for bacterial cells. J. Microbiol. Meth. 11:255-260.

A method was developed to use an ordinary spectrophotometer for obtaining the refractive index increment of bacterial cells, enabling determination of their solids content by immersion refractometry. The results agreed well with values for cells of *Serratia marcescens* obtained previously by two other methods.

Belliveau, B.H., T.C. Beaman, and P. Gerhardt. 1990. Heat resistance correlated with DNA content in Bacillus megaterium spores. Appl. Environ. Microbiol. 56:2929-2921.

Two subpopulations of *Bacillus megaterium* spores (1.360 and 1.355 g/ml) were obtained by density gradient centrifugation. The heavier spores had a higher thermoresistance (e.g., D_{80} = 186 versus 81 min) and a higher DNA content (1.25×10^{-14} versus 0.65×10^{-14} g per spore, apparently corresponding to digenomic versus monogenomic spores). No appreciable differences were found in the mineral and dipicolinic acid contents or in the inactivation kinetics of the two subpopulations. The implications of the findings are discussed with regard to mechanisms of heat resistance and of inactivation.

Marquis, R.E., and G.R. Bender. 1990. Compact structure of cortical peptidoglycans from bacterial spores. Can. J. Microbiol. 36:426-429.

Cortical peptidoglycans isolated from *Bacillus megaterium* ATCC 19213 and *Bacillus cereus* strain terminalis by chemical extraction of decoated spores were found to have few free amino groups, indicative of a high level of peptide cross-linking, low mineral contents, indicative of low levels of net negative charge, and low dextran-impermeable volumes, indicative of compact structures. Moreover, they had little capacity to swell or shrink in response to changes in environmental pH value. Overall, the data indicated that the cortical peptidoglycan sacculus is designed to serve primarily as a restraining structure rather than as a contractile or expansive element.